

A Review on Tumor Heterogeneity and Evolution in Multiple Myeloma: Pathological, Radiological, Molecular Genetic, and Clinical Integration

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Abstract

Recent research has dramatically advanced our understanding of the genetic basis of multiple myeloma (MM). MM displays enormous inter- and intratumoral heterogeneity, and underlies a clonal evolutionary process driven and shaped by diverse factors such as clonal competition, tumor microenvironment, host immunity, and therapy.

Two main cytogenetic groups are distinguished: MM with recurrent translocations involving the immunoglobulin heavy chain locus, and MM with hyperdiploidy involving the odd chromosomes. The disease virtually always starts with a preneoplastic prodromal phase – monoclonal gammopathy of undetermined significance – that variably progresses to symptomatic MM within a few months or many years. Tumor heterogeneity and its evolution in space and time have important consequences for the clinical management and outcome of MM patients. At diagnosis, spatial intratumoral heterogeneity poses a challenge for classification and risk stratification. During maintenance therapy, clonal evolution may complicate disease monitoring and promote drug resistance. Upon progression or transformation, identifying the dominant disease-driving neoplastic clones and elucidating their properties are key to tailor personalized therapy.

In this review, we discuss tumor heterogeneity and clonal evolution in MM, integrating pathological, radiological, molecular genetic, and clinical data. Current and prospective classification schemes and prognostic parameters, incorporating new genetic and proteomic discoveries and advances in imaging, are highlighted. In addition, the roles of the tumor microenvironment, host immunity, and resistance mutations, and their effects on therapy, are discussed.

An improved understanding of high-risk disease, tumor heterogeneity, and clonal evolution will guide future therapies and may ultimately lead towards a cure for MM.

Introduction

Plasma cell neoplasms (PCNs) arise from terminally differentiated antibody-producing B cells and evolve through a chain of genetic events from the indolent, pre-malignant disorder monoclonal gammopathy of undetermined significance (MGUS) to multiple myeloma (MM) and its end-stage variants. MM is the second most common hematological malignancy, affecting >90,000 individuals in the United States and representing 1.6% of all cancers [1]. The clinical behavior of MM and other advanced PCNs is very heterogeneous, with survival rates ranging from months to decades [2]. This heterogeneity can be attributed to tumor genetics and epigenetics, as well as a range of extrinsic factors including the tumor microenvironment (TME), disease stage, and host immune response [3-9]. The diagnosis and classification of PCNs is based on integration of clinical, serological, pathological, radiological, and molecular genetic findings. PCNs are categorized as precursor lesions, i.e. MGUS and smoldering MM (SMM); solitary plasmacytoma with osseous or extraosseous localization, standard-risk MM, and high-risk disease including advanced and relapsed/refractory (R/R) MM [10]. Furthermore, in the WHO classification, specific subtypes of PCNs are recognized based on their predominant clinical symptoms, such as AL amyloidosis; their association with specific syndromes, such as POEMS (**P**olyneuropathy, **O**rganomegaly, **E**ndocrinopathy, **M**onoclonal gammopathy and **S**kin changes); or their anatomic location, as for extramedullary plasmacytoma (EMP) and plasma cell leukemia (PCL).

New treatment strategies including proteasome-targeting drugs, monoclonal antibodies (mAbs), immunomodulators and autologous hematopoietic stem cell transplantation (auto-transplant) have induced long-term remissions and improved the outcomes of many MM patients. On average, fit elderly patients have a median overall survival (OS) of 5 years, and OS in younger patients receiving auto-transplant approaches 10 years [11]. However, for the 20-30% of MM patients with high-risk disease, even the most intensive therapies have not resulted in satisfactory outcomes [10, 12]. In these patients, tumor heterogeneity and clonal

evolution of MM under therapy favor the emergence of resistant clones that lose their dependence on the bone marrow (BM) microenvironment, ultimately relapsing as end-stage EMD or PCL [10]. Therefore, identifying high-risk patients and treating them accordingly is key to improve global outcomes in MM. This can be achieved by correct interpretation and integration of diagnostic findings from multiple clinical disciplines, including pathology, radiology and clinical genomics.

Recent sequencing efforts have shed light on the mutational landscape of PCNs and have unraveled a complex picture of initiation, evolution, and progression of these diseases, leading to inter- and intratumoral heterogeneity (**Figure 1**). Combining advanced medical imaging modalities with targeted biopsies and genetic investigation has provided insights into spatial intratumoral heterogeneity and regional genetic variation, uncovering the primary existence – or drug-induced emergence – of high-risk, disease-driving subclones that determine prognosis. These findings have raised new challenges for PCNs with regard to initial diagnosis and classification, as well as for follow-up protocols under therapy [10], and raise the following important questions: (1) What practical impact have pathological and genetic heterogeneity and clonal evolution on the clinical management of patients with PCN? (2) What is the role of advanced imaging modalities, i.e. diffusion-weighted magnetic resonance imaging (DW-MRI) and ¹⁸fluoro-deoxyglucose positron emission tomography / computed tomography (FDG-PET/CT) in routine PCN diagnosis and follow-up? (3) How does tumor heterogeneity as seen in these imaging modalities, such as large focal lesions (FLs) or EMD, influence downstream diagnostic procedures including guided lesion biopsies and extended sequencing? (4) What is the best way to integrate pathological, radiological and genetic findings (e.g. from multiple heterogeneous lesions), and what is their impact on prognosis and prediction of therapy response?

In this review, we will address these questions from a multidisciplinary point of view, integrating current knowledge on the biology, pathology, genetics, imaging, and clinical features of PCNs with a special focus on the impact of tumor heterogeneity.

Heterogeneity at the pathological level

According to the Revised 2017 WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues, PCNs are subdivided into 5 categories: (1) non-IgM MGUS; (2) plasma cell myeloma/MM, (3) solitary plasmacytoma; (4) monoclonal immunoglobulin (Ig) deposition disease; and (5) PCNs with associated paraneoplastic syndrome [13]. Trephine BM biopsies and aspirates continue to be pivotal for a correct diagnosis of PCNs. BM aspirates are often helpful to assess cytological abnormalities, but can sometimes be negative for plasma cells due to the heterogeneous distribution in the BM. Sheets of malignant plasma cells displaying cellular and nuclear atypia including multinucleation and prominent nucleoli, in combination with positivity for CD138, light chain restriction, and aberrant expression of CD56 in immunohistochemistry (IHC), render the diagnosis of MM. However, early cases with low percentages of neoplastic plasma cells are more challenging, and a diagnosis of MM requires the integration of clinical features, especially the presence or absence of CRAB criteria (hyperCalcemia, Renal insufficiency, Anemia and Bone lesions) (**Table 1**). In these early stages, morphological findings such as the dissociation of groups of plasma cells from their usual perivascular BM niche or signs of active bone resorption can provide a hint at the diagnosis. Prominent nucleoli, intracytoplasmic and intranuclear immunoglobulin inclusions (Russell bodies, Dutcher bodies, respectively) and deposits in macrophages (Gaucher-like cells or crystalline inclusions, respectively) may point to malignancy but may also be found in reactive processes and are therefore not specific. In addition to their diagnostic utility, morphology and IHC deliver valuable information with regards to potential biological and molecular genetic mechanisms driving the PCN. For example, MM with lymphoplasmacytoid

morphology is frequently associated with t(11;14)(q13;q32) translocation, exhibits staining for cyclin D1 in IHC and is commonly CD20-positive, potentially even resulting in a misdiagnosis of mantle cell lymphoma [14, 15] (**Figure 2**). Strong and homogeneous nuclear cyclin D1 positivity can be taken as a surrogate for the presence of a t(11;14) translocation, whereas more heterogeneous and weaker reactivity is observed mainly in cases with trisomy 11 [15]. In MGUS, cyclin D1 expression in plasma cells can confirm the presence of a clonal proliferation. Rare heavy chain isotypes are also associated with specific features; e.g. 20% of PCNs with lymphoplasmacytoid morphology are IgD-producing, whereas the rare IgM-producing MM shows conventional morphology and is CD20-negative, despite containing the t(11;14) translocation in >80% of cases [16-18]. Further cytomorphological features include mature, intermediate, immature and plasmablastic morphology, the latter associated with significantly worse prognosis and common *MYC* translocations, which usually represent a secondary genetic event in MM. Of note, plasmablasts and plasmablastic myeloma have been defined by different criteria cytologically (i.e., in the BM aspirate) and histologically (i.e. in the BM trephine biopsy) [18-20]. Cytologically, Greipp et al. defined plasmablasts as having a large central nucleus (>10 µm diameter) or a large nucleolus and scant cytoplasm; for a diagnosis of plasmablastic myeloma, plasmablasts needed to constitute $\geq 2\%$ of nucleated marrow cells in the aspirate. In contrast, Bartl et al. characterized plasmablastic myeloma as having a majority of cells with immunoblast-like features with prominent central nucleoli and rather scant cytoplasm in the BM trephine. Irrespective of these different diagnostic criteria, plasmablastic myeloma usually accounts for 5-15% of MM cases and shows aggressive behavior.

The phenotype and phenotypic variability of malignant plasma cells is also exploited in flow cytometry, which is a mainstay in the diagnostic evaluation and especially minimal residual disease detection of MM. In addition, the antigen profile of clonal plasma cells has been used for prognostic purposes and for risk assessment in MGUS and SMM [21-25].

Another aspect of heterogeneity at the pathological level are solitary plasmacytomas, accounting for about 6-10% of all PCNs, which manifest either as solitary osteolytic lesion or as a tumoral proliferation in an extramedullary location, mostly mucosal sites in the head and neck region, less commonly in lymph nodes. Although extramedullary plasmacytomas (EMPs) by definition do not show BM involvement, sensitive techniques can detect clonal BM plasma cells in the majority of patients with EMP [26]. Furthermore, EMPs are very similar to MM both phenotypically and genetically, with the exception that EMPs are usually negative for the t(11;14) translocation and markers of advanced disease, such as *MYC* translocations or *TP53* alterations [27, 28]. Despite this close relationship to MM, EMPs shows a good prognosis with local radiotherapy alone, and only about 15% of patients progress to systemic disease. This underlines the importance of non-genetic factors for disease progression. From the practical point of view, separation of generally indolent EMPs from aggressive extramedullary disease is of great importance, and usually relies on the clinical circumstances and certain pathological features such as cellular maturity and the identification of markers of aggressive disease (**Figure 2**).

MM focal lesions (FLs) are localized tumor cell accumulations that are present in the majority of MM patients at diagnosis and are detectable by advanced imaging modalities (**Figure 3**). FLs represent mutational hotspots consistent with the regional outgrowth of advanced tumor clones [29]. Detailed histopathological analyses on the cellular composition and spatial organization of MM FLs are largely lacking, mainly because of their frequently difficult localization and accessibility for radiological guided biopsy. Tissue from FLs can be obtained during surgery for pathological fractures, and preliminary studies on these FLs compared to matched random BM biopsies did not show significant differences in the proliferation rate of malignant plasma cells (L.R. and N.W., unpublished observations). However, systematic studies addressing the FLs' tissue characteristics, including mapping their

immune tumor microenvironment, have not yet been performed, and there are surprisingly limited data on osteolytic lesions.

In summary, MM shows significant heterogeneity at the pathological level, and the pathologist can use morphological cues and IHC to guide downstream analyses, such as genetic testing. Future pathology studies in MM should focus on, and describe in detail, the spatial organization of FLs.

Heterogeneity at the molecular genetic level

MM is characterized by extensive inter-patient heterogeneity [30]. Yet, it can be broadly categorized into two groups: those with Ig heavy chain gene translocations involving chromosome 14q32 (including, in descending order of frequency, t(11;14), t(4;14), t(14;16), t(14;20) and t(6;14)); and those with hyperdiploid genomes (i.e., trisomies of odd-numbered chromosomes with the exception of 1, 13 and 21). Additional gross chromosomal aberrations, such as deletions and amplifications, are often observed, leading to emergence of new subclones and intratumoral heterogeneity. To better understand intratumoral heterogeneity, we recently sequenced several MM samples from the same patient, including iliac crest and radiology-guided FL biopsies. Spatial genomic heterogeneity at the chromosomal and mutational level, where somatic aberrations were present at one site but absent at another (private mutations), was observed in the majority of patients. On average, three unshared copy number aberrations (CNAs) between paired samples were found in these patients. These included spatial differences in commonly used prognostic markers such as del(17p), *MYC* translocations, and aberrations of chromosome 1. In contrast, initiating events such as t(11;14) and t(4;14) were uniformly shared among investigated regions, consistent with their role as early disease drivers. In addition, mutational analysis revealed that private mutations were found in 76% of newly diagnosed MM (NDMM) patients [29]. The non-homogeneous distribution of high-risk clones and potentially targetable driver mutations pose a significant

challenge for correct risk classification and effective therapy [29, 31, 32]. The prime, clinically relevant example of spatial heterogeneity discovered in this study was in a patient who had an ultra-high-risk clone in a biopsy from the lumbar spine that had a high-risk score according to gene expression profiling (GEP) data, a bi-allelic *TP53* deletion and an aberration of chromosome 1p, all of which were absent in the iliac crest biopsy. In contrast, the iliac crest biopsy was GEP low-risk, had a hyperdiploid karyotype and a *BRAF*^{V600E} mutation.

A recent investigation of 1273 NDMM patients yielded profound insight into the interplay of commonly mutated genes. Walker et al. found significant associations between certain translocations, oncogene or tumor suppressor gene mutations, chromosomal copy number changes, and hyperdiploidy. Examples of such events included t(11;14) in combination with mutations in *CCND1* and *IRF4*; t(4;14) in combination with mutations in *FGFR*, *DIS3* and *PRKD2*; and hyperdiploidy in combination with gain 11q, mutations in *FAM46C* and *MYC* rearrangements, amongst others [33]. Some of these mutations, such as those in *CCND1* and *FGFR*, are probably the effect of aberrant somatic hypermutation targeting translocated genes. Furthermore, the authors observed a significant inverse correlation between the number of oncogenic driver mutations per sample and progression free as well as overall survival. In addition, this study found novel potentially targetable mutations in the epigenetic regulators *IDH1* and *IDH2*, and identified associations between certain *BRAF* mutations and chromosomal translocations, i.e. t(14;16) and *BRAF*^{D594N}, and t(4;14) and *BRAF*^{V600E} [33]. The latter finding is clinically relevant since BRAF inhibitors are selective for *BRAF*^{V600E} and should not be used in patients with *BRAF*^{D594N} or RAS mutations [31, 34, 35].

Heterogeneity at the radiological level

MM intra- and intertumoral heterogeneity are clearly reflected at the radiological level. While some patients present with a diffuse plasma cell BM infiltration only, up to 75% of patients show FLs, localized accumulations of tumor cells in the skeletal system (**Figure 3**). Number,

size, location in the skeleton (axial vs. appendicular), type (intra-, para- or extramedullary), and metabolism of FLs differ between patients, dramatically increasing the extent of inter-patient heterogeneity in MM. The two main imaging techniques used for disease detection are ^{18}F -fluorodeoxyglucose positron emission tomography/computed tomography (FDG-PET/CT) and diffusion weighted magnetic resonance imaging (DW-MRI). While FDG-PET/CT is based on the quantification of increased glucose uptake by tumor cells, in DW-MRI cellularity is measured by quantifying the diffusion of water molecules and the microcirculation of blood in the capillary network [36, 37]. FDG-PET/CT and DW-MRI have comparable diagnostic accuracy, and DW-MRI has the advantage of not exposing the patient to ionizing radiation [38-40].

Clinically, the focus has been on the number and type of FLs. Both, EMD and more than 3 FLs in FDG-PET/CT negatively impact outcome [41-46]. Another variable that seems to impact prognosis is the location of FLs: Abe et al. recently showed that MM patients with multiple FLs in the appendicular skeleton suffered from poor outcome [47]. Combining whole-body functional imaging and data from multi-region sequencing, we identified the size of FLs as an important variable [29, 46]. We demonstrated that spatial differences were mainly seen in patients with large (>2.5 cm) FLs, suggesting that clinical imaging can be used to identify patients with extensive intratumoral heterogeneity. Indeed, using the size of FLs as a surrogate marker for the level of intratumoral heterogeneity, we showed that the presence of multiple large FLs was an independent adverse prognostic factor in MM [46]. Accounting for the size of FLs, more than 3 FLs in FDG-PET/CT were not prognostically relevant anymore.

We have shown that highly advanced clones can be restricted to an FL [29]. Since response to treatment could be different at that location compared to other sites in the BM, the question becomes whether iliac crest sampling is sufficient for disease monitoring during treatment. We and others have recently tested the value of imaging as an alternative method for detection of minimal residual disease (MRD) [25, 48]. Using the two functional methods, DW-MRI and

FDG-PET/CT, we showed that one fourth of NDMM patients still had residual FLs when they achieved complete remission (CR). Combining flow cytometry for MRD detection and the two imaging methods, we demonstrated that less than 10% of MRD-negative NDMM patients in CR still presented with residual FLs. In contrast, ~50% of heavily pretreated patients who achieved MRD-negative CR during salvage therapy presented with FLs, illustrating the value of functional imaging in these patients [49]. Last but not least, the spleen signal on DW-MRI also provides prognostic information, in that patients lacking spleen signal usually show high tumor burden and poor prognosis, especially when suffering from high-risk disease [50].

Role of the BM microenvironment and host immunity in MM heterogeneity

Recent evidence indicates that intratumoral heterogeneity can be influenced by clonal competition, clonal cooperativity, pH, oxygenation, and the BM microenvironment (BMME) [4-6, 9, 51, 52]. The role of the BMME in MM pathogenesis has been extensively reviewed [53-57]. The BM is a complex organ consisting of numerous highly specialized cell lineages that orchestrate blood production, immunity, and skeletal integrity over a lifetime of an organism. Because mature, long-lived plasma cells primarily reside in the BM, the BMME is key in the development and progression of MM (**Figure 4**) [54]. As an immunological organ by itself, the BM hosts a wide range of immature and mature innate and adaptive immune cell types [58]. To accommodate its high metabolic needs, the BM is also richly vascularized with different types of arterioles, transitional capillaries, and sinusoids [59]. Besides these cellular compartments, which also include mesenchymal cells such as BM stromal cells, adipocytes and osteolineage cells, the BMME consists of solid extracellular matrix components (such as collagenous bone) and a liquid milieu containing growth factors, cytokines and chemokines. Immune, stromal, osseous and endothelial cells all contribute to this milieu, which promotes survival, proliferation, and drug resistance of MM cells [56]. By secreting regulatory cytokines such as transforming growth factor (TGF)- β and interleukin (IL)-10, MM cells induce a local

and systemic immunosuppression that prevents host antitumoral immunity and contributes to an increased susceptibility to infections [60]. In addition, MM cells produce angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin-1 and fibroblast growth factor (FGF), which contribute to the enhanced BM angiogenesis observed in MM patients [55, 61]. Interestingly, MM cells also produce exosomes that carry multiple angiogenic factors and directly promote endothelial cell growth [62]. Of note, Nakamura et al. recently demonstrated that IL-18, produced by multiple cell types in the BMME, drove the generation of MM-supporting myeloid-derived suppressor cells (MDSCs) that negatively impacted antitumoral CD8⁺ T cell responses, and showed that high IL-18 levels in BM plasma were associated with poor outcome in MM patients [63]. Indeed, BM MDSCs were already reported earlier to play a pivotal role in MM pathogenesis [64]. In addition to cytokines and growth factors, MM cells express adhesion factors such as CD38 that allow them to adhere to BM endothelial and stromal cells. BM stromal cells in turn up-regulate the expression of IL-6 and other cytokines that activate osteoclasts (OCs) and drive MM cell proliferation [53]. Interestingly, a study by Frenquelli et al. showed that ROR2, a receptor for the non-canonical WNT pathway, is essential for MM cell interactions with the BMME, and that ROR2 inhibition led to MM cell detachment resulting in apoptosis [65]. OCs, bone resorbing cells that are formed as giant syncytia by the fusion of monocytes, are mainly responsible for the MM-specific bone lesions and the increased risk of fractures. OC numbers and activity are strongly enhanced in MM, and OCs directly protect MM cells from immune action by CD4⁺ and CD8⁺ T cells [66]. Numerous additional BMME cell types including osteoblasts, macrophages and plasmacytoid dendritic cells were shown to contribute to MM pathogenesis [67-69]. Many currently available MM therapies, including immunomodulators and bisphosphonates, exert their functions at least in part by affecting non-tumor cells in the BMME [56]. However, insights into the mechanisms by which the BMME supports or even promotes MM heterogeneity are almost completely lacking. An important factor for the establishment of intratumoral heterogeneity is the ability of MM cells

to mobilize into the bloodstream and re-enter the BM at different sites. The chemokine receptor CXCR4, a key mediator of hematopoietic stem cell retention in the BM, also plays a role in the interactions of MM cells with the BMME. Inhibiting CXCR4 with the drug plerixafor (AMD3100) disrupted these interactions, mobilized MM cells into the periphery and increased their sensitivity to multiple therapeutic agents [70].

In summary, the BMME and host immunity both are crucial factors in MM pathogenesis. Future studies should address how they contribute to MM genetic heterogeneity and why end-stage diseases such as EMD and PCL lose their dependence on microenvironmental survival signals. Answering these important questions will improve our understanding of MM in general and pave the way for future therapies.

Current therapy and the impact of tumor heterogeneity

Although molecular segmentation strategies have shown the extent of tumor heterogeneity in MM, the standard clinical approach is still more akin to “one size fits all”. Younger individuals and medically fit patients aged up to 75 years undergo induction therapy utilizing at least one of the so-called novel agents lenalidomide or bortezomib, followed by peripheral stem cell apheresis, high-dose melphalan therapy, auto-transplant, and lenalidomide maintenance therapy. In some centers, a second high-dose therapy with auto-transplant 2-6 months after the first transplant (“tandem”) is preferred over a single transplant. The standard dose of melphalan is 200 mg/m²; in elderly or frail patients the dose is frequently reduced to 140 mg/m². Consolidation therapy after auto-transplant is another strategy to improve depth of response, and probably outcomes. The efficacy of these additional elements is still a matter of debate [71]. However, *Total Therapies*, which include multi-agent induction therapy, two auto-transplants, consolidation, and intensified maintenance have produced the best reported outcomes so far with some patients even considered to be cured [72]. Treatment for transplant-

ineligible patients typically consists of doublet or triplet drug regimens, which are frequently combined with daratumumab, an anti-CD38 monoclonal antibody [73, 74].

Only few studies have investigated specific treatments for GEP-defined high- and low-risk disease [75, 76]. Unfortunately, these studies were not successful and could neither support intensification in high-risk nor de-escalation in low-risk patients. In a study by Cavo et al., patients with high-risk disease, defined there as international staging system (ISS, **Table 2**) stage II+III, high-risk cytogenetics and failure to achieve CR, benefited from tandem transplant as compared to single transplant (median progression-free survival: 35 vs. 14 months). However, overall survival in the high-risk group was still clearly inferior when compared to standard-risk patients. These results suggest that double-transplant delays disease progression in high-risk patients but does not overcome high-risk disease *per se* [77]. In summary, current clinical practice usually does not take into account tumor heterogeneity, with a few exceptions, which are discussed below, and recapitulated in **Table 3**.

Treatment with venetoclax, an oral BH3-mimetic, in patients harboring translocation t(11;14) is a good first example for individualized MM therapy utilizing the distinct susceptibility of this molecular subtype to BCL-2 inhibition. Tumor cells with t(11;14) show a more B cell-like phenotype and more likely express high levels of BCL-2 relative to BCL-X_L and MCL-1. Evaluating single-agent venetoclax in R/R MM, the overall response rate was 21%, and most responses (86%) were seen in patients with t(11;14) translocation, with 27% of these patients achieving a very good partial response (VGPR) or better [78]. Combining venetoclax with bortezomib and dexamethasone the overall response rate was 67% in patients with R/R MM, and 42% of patients achieved a VGPR or better [79]. Response was not limited to cases with t(11;14), but best overall response rate was observed in patients with high BCL-2 expression (94%). Notably, patients with bortezomib-sensitive disease were more likely to respond than those whose disease was refractory to prior bortezomib, suggesting that

venetoclax cannot fully overcome bortezomib resistance [80]. In summary, treatment with venetoclax in patients with t(11;14) represents the prime example for personalized MM therapy. However, venetoclax is not yet officially approved for the treatment of MM by the United States Food and Drug Administration (FDA), and it is not mentioned in the National Comprehensive Cancer Network (NCCN) guidelines for MM [81].

Another good example is the impact of proteasome inhibitors on t(4;14)-positive MM, a subgroup with dismal outcomes before the introduction of this class of drugs. At the University of Arkansas for Medical Sciences in Little Rock, Dr. Barlogie and team treated patients with several generations of *Total Therapy* encompassing multi-agent induction therapy, two auto-transplants, and intensified maintenance. Bortezomib was introduced in *Total Therapy 3a* in the induction, consolidation and maintenance phase of therapy. A survival benefit was mainly seen for patients with t(4;14), highlighting a favorable effect of proteasome inhibition particularly in this subgroup [12]. Avet-Loiseau et al. reported on 507 NDMM patients treated with bortezomib/dexamethasone induction followed by transplant and also observed significantly improved overall survival for t(4;14) positive cases [82]. In summary, proteasome inhibitors improve outcomes in patients with t(4;14). Bortezomib maintenance is one option recommended in the NCCN guidelines for MM [81].

The third example for personalized therapy is the use of vemurafenib and other BRAF and MEK inhibitors in *BRAF*^{V600E} mutated MM [83-85]. Mutations at this location occur in >5% of NDMM and the frequency increases from baseline to relapse [83, 86]. Raab et al. and others have reported on patients achieving CR after BRAF targeted therapy in R/R MM, and a phase 2 trial (BIRMA trial, NCT02834364) is currently evaluating this concept in a larger number of patients [83]. However, although being the proof of concept for targeting actionable mutations, duration of response is often short and patients relapse with *BRAF*^{V600E} negative clones [31]. Moreover, mutations in genes involved in the MAP kinase pathway are subclonal in the majority of patients [31, 87] and spatial genomic heterogeneity has been reported for

these potential targets [29]. Together, targeting mutations in MM may be an approach for R/R patients with limited therapeutic options. However, we recommend genomically characterizing these patients and discussing the results in the framework of a molecular tumor board.

Conclusions

Recent research has uncovered a striking amount of inter- and intratumoral heterogeneity in PCNs at all levels of investigation. Despite our increased understanding of PCNs and better therapies, which have resulted in an improved outlook for the majority of patients, spatially distributed genetic and biological heterogeneity with outgrowth of aggressive and therapy-resistant subclones still preclude long-term cure in most patients. The combination of advanced imaging modalities combined with targeted genetic investigation of FLs may lead to better risk estimation and early identification and targeting of aggressive subclones. Yet, how the ever more complex personalized MM medicine can be harmonized with increasing economic pressures and patient dignity is a challenge for the future.

Tables

Table 1: WHO definition of MM

Parameter	Diagnostic criterion
1. Clonal plasma cell neoplasm (clonality proven by IHC, IF or flow cytometry)	
Clonal BM plasma cells	> 10%
OR	
Plasmacytoma	biopsy-proven
AND	
2. Any one of the following myeloma-defining events	
Any one of the following criteria of end-organ damage (CRAB -criteria)	HyperCalcemia (>0.25 mmol/L above normal or > 2.75 mmol/L)
	Renal insufficiency (creatinine clearance <40 ml/min or serum creatinine > 177 µmol/L)
	Anemia (hemoglobin > 20 g/L below normal, or < 100 g/L)
	Bone lesions (one or more osteolytic lesions on skeletal radiography, CT, or ¹⁸ F-FDG-PET/CT)
OR	
Any one of the following biomarkers of malignancy	<ul style="list-style-type: none"> • Clonal BM plasma cells ≥ 60% • Involved-to-uninvolved serum free light chain ratio ≥ 100 (involved free light chain must be ≥ 100 mg/L) • >1 focal lesion on MRI (each ≥ 5mm in size)

Adapted from [13, 88]

Table 2: International Staging System (ISS) and Revised ISS (R-ISS) for MM

Parameter	ISS stage I	R-ISS stage I
	all of the following:	all of the following:
Serum albumin	≥ 35 g/L	≥ 35 g/L
Serum β ₂ -microglobulin	< 3.5 mg/L	< 3.5 mg/L
Serum lactate dehydrogenase	not considered	normal
Cytogenetics	not considered	non-high-risk: absence of t(4;14), t(14;16), del(17p)
	ISS stage II	R-ISS stage II
ISS / R-ISS stage	not ISS stage I or III	not R-ISS stage I or III
	ISS stage III	R-ISS stage III
Serum β ₂ -microglobulin	≥ 5.5 mg/L	≥ 5.5 mg/L
		AND
Serum lactate dehydrogenase	not considered	elevated
		OR
Cytogenetics	not considered	high-risk: presence of t(4;14), t(14;16) or del(17p)

Adapted from [2, 89]

Table 3: Molecular abnormalities, their effects, prognostic impact, and candidate drugs / therapeutic implications

Molecular abnormality	Effect / function	Prognostic impact	Candidate drugs / therapeutic implications	References
t(4;14) (10-15% of patients)	Overexpression of MMSET and FGFR3; increased histone H3K methylation, up-regulation of CCND2	Adverse prognosis	Bortezomib (improves survival)	[1, 12, 30, 81]
t(6;14) (~1% of patients)	Overexpression of CCND3 leading to malignant trans-formation	Neutral	Palbociclib (CDK4/6 inhibitor)	[1, 12, 30] NCT03297606 NCT02465060 NCT02693535 NCT00555906 NCT02030483
t(14;16) (3-5% of patients)	Overexpression of MAF leading to up-regulation of CCND2, “APOBEC signature” (increased mutation rate)	Adverse prognosis (neutral in a large study with 1004 patients [90])	-	[1, 12, 30, 90, 91]
t(11;14) (15-20% of patients)	Overexpression of CCND1 under IgH promoter leading to malignant trans-formation; proliferation; dependence on anti-apoptotic proteins	Neutral (co-occurrence with activating CCND1 mutations in 10% associated with adverse prognosis)	BH3-mimetics (e.g. venetoclax) MCL1-inhibitors (e.g. MIK665, AMG397) Palbociclib (CDK4/6 inhibitor)	[79, 80] [92] NCT02992483 NCT03465540 NCT03297606 NCT02465060 NCT02693535 NCT00555906 NCT02030483
t(14;20) (~1% of patients)	Overexpression of MAFB leading to up-regulation of CCND2, “APOBEC signature” (increased mutation rate)	Adverse prognosis	-	[1, 12, 30, 91]
ADAR1 over-expression (due to 1q21 amplification present in up	Transcriptome hyper-editing	Disease progression and aggressiveness	8-Azaadenosine (could be a candidate drug to inhibit ADAR1)	[93]

to 45% of patients)			Resistance to PD-1 immune check-point blockade	[94]
Mutations in BRAF (6-12% of patients), KRAS (~20% of patients), NRAS (~20% of patients)	MAPK pathway activation	Associated with disease progression (mutations often subclonal)	Encorafenib Binimetinib Trametinib Selumetinib Vemurafenib	[31, 83-86] NCT02834364 NCT01989598 NCT01085214 NCT01524978
MAF over-expression (30% of patients)	Alteration of integrin beta 7, up-regulation of CCR1	Increased BM homing and survival in the BMME	-	[30]
MYC over-expression (secondary translocations, in 15-20% of patients)	Kataegis (localized hypermutation pattern linked to APOBEC deregulation)	Adverse prognosis	CPI-0610 (BET inhibitor)	NCT02157636
SK2 over-expression (not correlated with cytogenetic abnormalities)	Production of S1P	Mitogenic and anti-apoptotic functions of S1P	ABC294640	[95] NCT02757326

Adapted in part from [1]. Abbreviations: ADAR1, adenosine deaminase acting on RNA 1; APOBEC, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like; BET, bromodomain and extra-terminal motif protein; BH3, BCL-2 homology domain 3; BM, bone marrow; BMME, BM microenvironment; CCND, Cyclin D; CDK, cyclin-dependent kinase; CCR1 C-C chemokine receptor 1; FGFR3, fibroblast growth factor receptor 3; MAF, V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog; MAP, mitogen-activated protein kinase; MCL-1, myeloid cell leukemia-1; MMSET, multiple myeloma SET domain; MYC, V-Myc Avian Myelocytomatosis Viral Oncogene Homolog; PD-1, programmed cell death protein-1; S1P, sphingosine-1-phosphate; SK2, sphingosine kinase 2

Figure legends

Figure 1. Tumor heterogeneity in MM. At the highest genomic level, inter-patient/intertumoral heterogeneity (top panel) can be viewed as patients having tumors with hyperdiploid genomes (~50%) or primary Ig heavy chain (IgH) translocations (~50%), with rare exceptions showing a combination of both. Moreover, there is a vast variability at the level of additional driver mutations between patients, as well as differences in the distribution of lesions and their histomorphological appearance, further contributing to intertumoral heterogeneity (not depicted). Intra-patient/intratumoral heterogeneity (bottom panel) can be described as the heterogeneous distribution of genetically distinct MM clones and subclones within a single patient. Intratumoral heterogeneity may complicate diagnostics and classification and represents a significant challenge for optimal personalized therapy.

Figure 2. Heterogeneity at the pathological level. (A-E) Extramedullary solitary plasmacytoma of the upper airways shows an infiltration by mature plasma cells with absence of significant atypia. (A-B) Giemsa staining, 100x and 400x magnification, respectively. (C-E) IHC for (C) MUM-1/IRF-4, (D) kappa (highlighting the malignant clone), and (E) lambda light chains (highlighting rare reactive plasma cells). Magnification, 400x. (F-I) Osteolytic lesion of anaplastic MM with large, immunoblast-like cells with narrow rims of basophilic cytoplasm and large central nucleoli, numerous atypical mitoses, and apoptotic bodies. (F) Hematoxylin & eosin (H&E) staining, (G) Giemsa staining. Magnification, 400x. (H-I) IHC for (H) MYC (only rare positive cells), and (I) Ki-67/MIB-1 (>80% positive cells). Magnification, 100x. (J-K) Medullary anaplastic MM a predominant population of small to medium sized cells and scattered cells with anaplastic morphology. (J) H&E staining, (K) IHC for p53 (homogenous and strong positive staining, indicative of p53-mutation). Magnification, 200x. (L-N) MM with lymphoplasmacytoid morphology of the BM. (L) H&E staining, (M) Giemsa staining.

Magnification, 200x. (N) IHC for cyclin D1 (marked overexpression, indicative of a chromosome 11 translocation). Magnification, 400x.

Figure 3. Heterogeneity at the radiological level. Up to 75% of myeloma patients present with FLs, and a high number and large size of FLs both are associated with an adverse prognosis. FDG-PET (left image) and DW-MRI (right image) of the same patient show heterogeneous disease involvement with multiple FLs. Some FLs are well visible in both imaging modalities (blue circles), whereas other FLs are much more pronounced in the DW-MRI (red circles). A possible explanation for these differences is metabolic heterogeneity, in that some FLs may express lower levels of hexokinase-2, impairing their uptake of glucose [36].

Figure 4. Role of BM microenvironment and host immunity in MM pathogenesis. MM cells produce immunosuppressive cytokines including IL-10 and TGF- β (center), directly inhibiting T cell responses. In addition, MM cells secrete angiogenic factors VEGF, FGF and ANGPT1 that contribute to BM angiogenesis (bottom left). Multiple cell types of the BMME produce IL-18, which supports the generation of MDSCs that inhibit CD8⁺ T cell responses (top left). The CXCL12-CXCR4 interaction is important for MM cell retention in the BMME. Furthermore, adhesion of MM cells to BMSCs, mediated in part by CD38, activates BMSCs to produce IL-6, which in turn stimulates OCs for increased bone resorption (bottom center). OCs also directly inhibit the proliferation of CD4⁺ and CD8⁺ T cells (top right). Blue arrows indicate stimulation, red arrows indicate inhibition. Abbreviations: ANGPT1, angiopoietin-1; BMME, bone marrow microenvironment; BMSCs, bone marrow stromal cells; CXCL12, C-X-C chemokine ligand type 12; CXCR4, C-X-C chemokine receptor type 4; FGF, fibroblast growth factor; IL, interleukin; MDSCs, myeloid-derived suppressor cells; MM cell, multiple myeloma

cell; OBs, osteoblasts; OCs, osteoclasts; TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor. Parts of this figure were created with BioRender.com.

ACCEPTED MANUSCRIPT

Compliance with ethical standards

The authors state that they comply with the ethical standards specified by Virchows Archiv.

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Conflicts of interest

The authors declare that no conflicts of interest exist.

Author contributions

All authors contributed to the writing and revision of the manuscript and accepted its final version.

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Figures

Figure 1

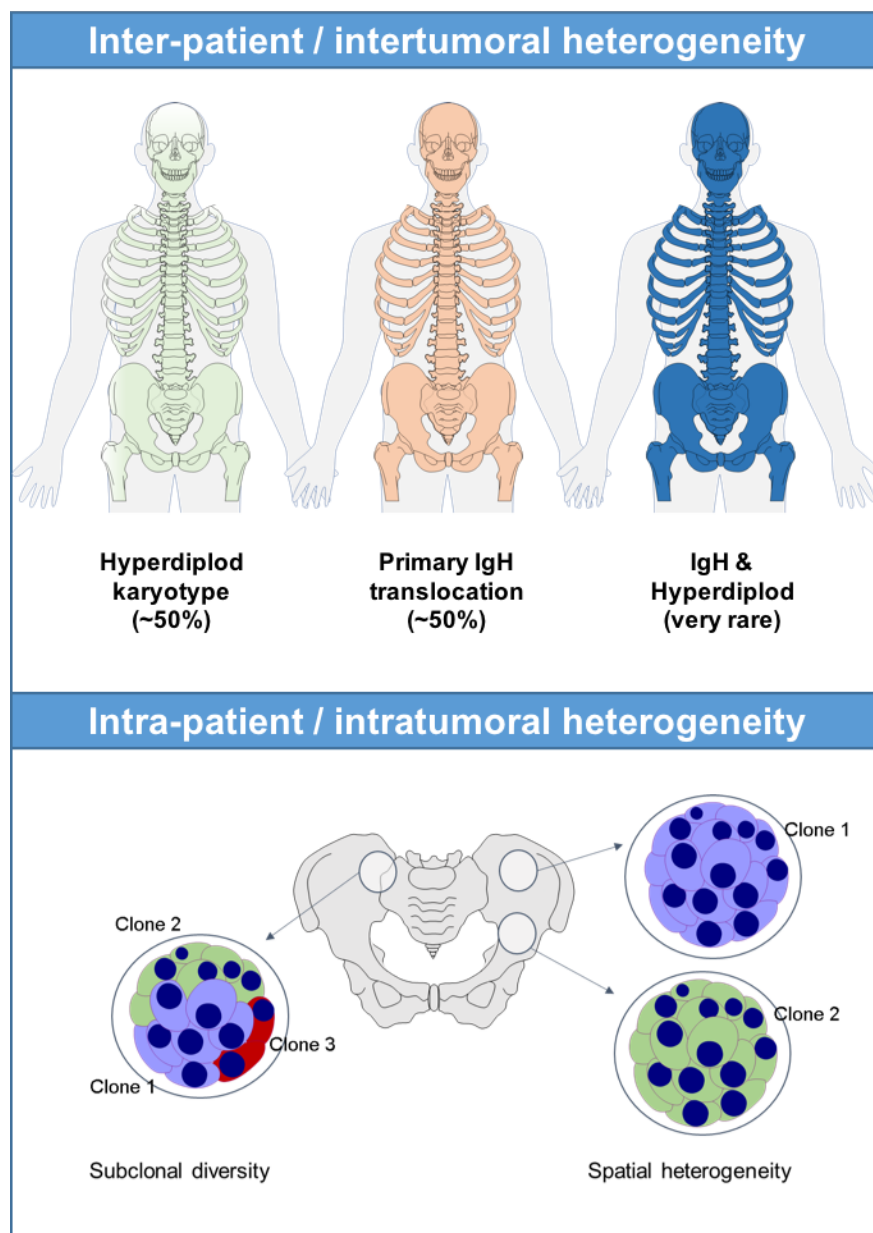


Figure 2

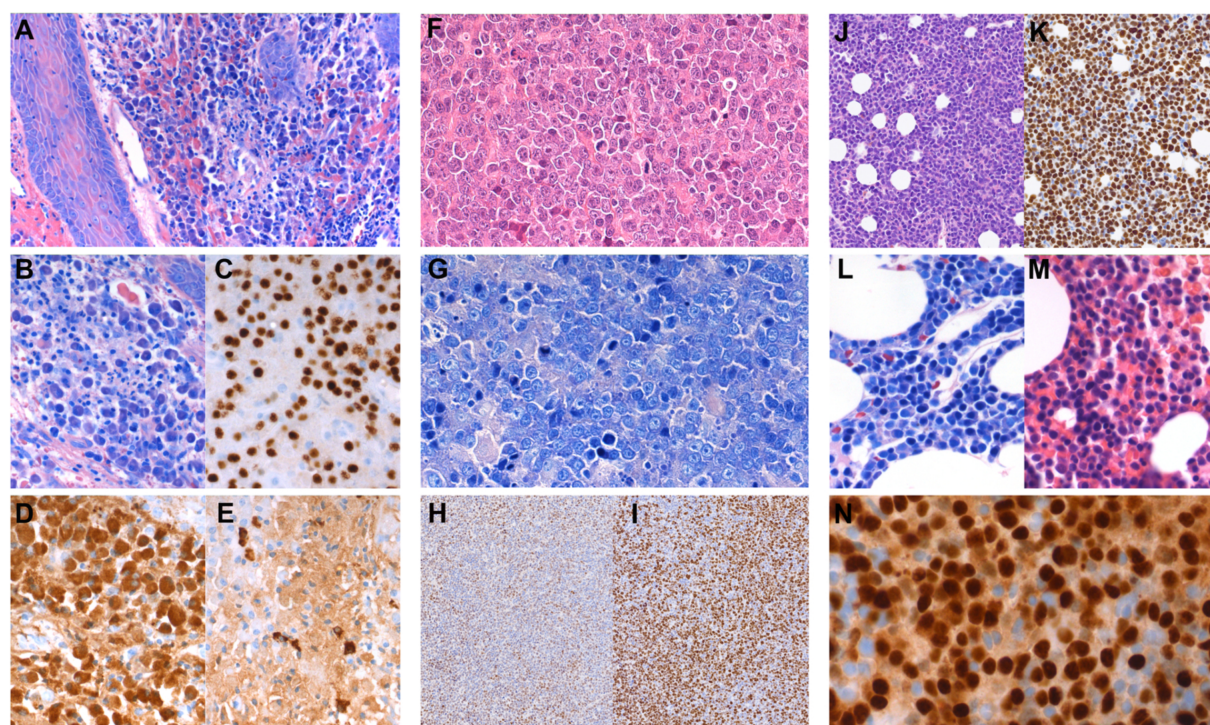


Figure 3

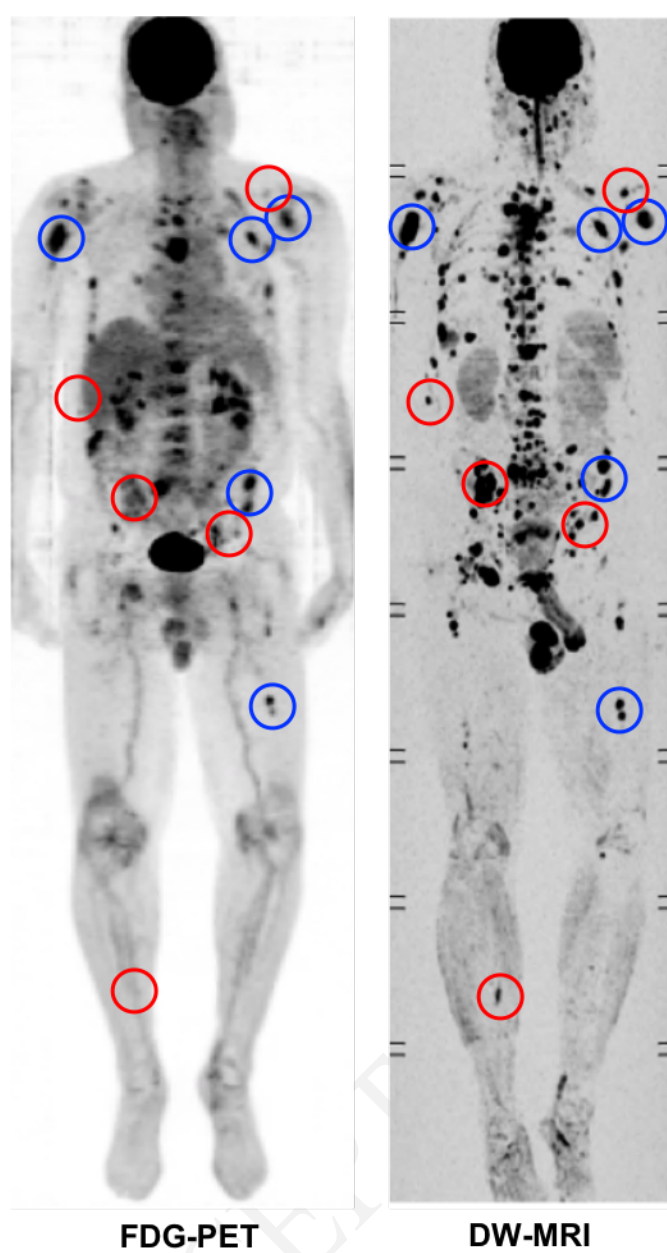


Figure 4

